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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 35/12, 35/28, 48/00, C12N 5/08, 5/10</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/14853</b> <b>(43) International Publication Date:</b> 23 May 1996 (23.05.96)
<b>(21) International Application Number:</b> PCT/US95/14773 <b>(22) International Filing Date:</b> 9 November 1995 (09.11.95) <b>(30) Priority Data:</b> 08/337,603 10 November 1994 (10.11.94) US <b>(71) Applicants:</b> UNIVERSITY OF WASHINGTON [US/US]; Suite 200, 1107 N.E. 45th Street, Seattle, WA 98101 (US). FRED HUTCHINSON CANCER RESEARCH CENTER [US/US]; 1124 Columbia Street, Seattle, WA 98104 (US). <b>(72) Inventors:</b> ALLEN, Margaret, D.; 319 W. Kinnear Place, Seattle, WA 98119 (US). ANDREWS, Robert, G.; 4640 N.E. 89th, Seattle, WA 98115 (US). <b>(74) Agents:</b> PARMELEE, Steven, W. et al.; Townsend and Townsend and Crew, One Market Plaza, Steuart Street Tower, San Francisco, CA 94105-1492 (US).	<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> INTRATHYMIC STEM CELL IMPLANTATION  <b>(57) Abstract</b>  Engraftment of grafts in transplant recipients are enhanced by implanting CD34-positive hematopoietic cells obtained from the graft donor in the thymus of the transplant recipient. The methods enhance engraftment of both allogeneic and xenogeneic transplants. Methods are also provided for supplying a gene product of interest in a host mammal by implanting in the thymus of the host CD34-positive hematopoietic progenitor cells which encode the gene product of interest. The CD34-positive hematopoietic cells can be genetically engineered to produce the gene product of interest in the recipient host.		

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## INTRATHYMIC STEM CELL IMPLANTATION

Background of the Invention

10 Mortality following cardiac transplantation has remained unchanged since 1981 in spite of many advances in immunosuppression. Complications attributable to immunosuppression (infection and malignancy) and to chronic rejection (graft coronary disease) together account for 90% of deaths following thoracic organ transplantation. Kaye, J. Heart Lung Transplant. 11:599-606 (1992). These problems are magnified in the infant and pediatric transplant populations where mortality due to rejection is significantly higher, coronary disease can develop within 6 months post-transplant (Pahl et al., J. Pediatr. 116: 177-183 (1990)), and transplant lymphoproliferative disease is more prevalent. Penn, J. Heart Lung Transplant. 12:S328-336 (1993). Additionally, in infant and adolescent transplant recipients, immunosuppression inhibits normal growth and development. Baum et al., Pediatrics 88:203-214 (1993). To improve long-term outcomes new strategies must be developed which could achieve freedom from graft rejection without standard immunosuppression, i.e. by developing tolerance to the graft.

30 The finding of low frequencies of circulating donor cells, microchimerism, in the peripheral blood of liver transplant recipients who were free from rejection suggests that hematopoietic microchimerism might correlate with tolerance to solid organ allografts. Starzl et al., Lancet 340:876-877 (1992) and Starzl et al., Lancet 339:1579-1582 (1992). This has been recently confirmed in twenty-one such liver recipients who have now been completely weaned off of immunosuppression. Ramos et al., Amer. Soc. Transplant Surg. May 1994, III-4. The corollary hypothesis, that the induction

of microchimerism might produce a state of tolerance, is now under intense investigation.

Hematopoietic chimerism can currently be produced in humans by allogeneic bone marrow transplantation following myeloablation. Concomitant allogeneic marrow and solid organ transplantation has been proposed as a method of producing chimerism and, perhaps, tolerance to the solid organ graft. This would entail the risks of myeloablative therapy and/or graft versus host disease. Unlike the grave prognoses with hematologic malignancies, the excellent early graft survival rates in cardiac transplantation are such that the risks incurred by reproducing full marrow transplant myelosuppressive strategies might override the benefits of chimerism.

What is needed in the art is a method of enhancing engraftment of donor solid organ, tissue and cell transplants that can be accomplished safely and 1) within current or reduced (rather than augmented) immunosuppressive regimens, 2) within the logistical constraints of transplantation procedures, and 3) minimizing the risk of graft versus host disease. Quite surprisingly, the present invention fulfills this and other related needs.

### Summary of the Invention

The present invention provides compositions and methods for enhancing engraftment of a graft in a transplant recipient. The graft is obtained from a donor who is different from the transplant recipient, i.e., allogeneic or xenogeneic. CD34-positive hematopoietic cells obtained from the donor are implanted in the thymus of the transplant recipient in amount sufficient to establish tolerance to the graft, thereby enhancing engraftment of the graft in the recipient. The CD34-positive hematopoietic cells may be obtained from the bone marrow or peripheral blood of the graft donor, and preferably are at least about 50% pure for CD34-

positive cells, more preferably at least approximately 98% pure. In some embodiments approximately  $5 \times 10^5$  to  $1 \times 10^8$  CD34-positive hematopoietic cells are implanted in the thymus of the transplant recipient. The implantation can be by way of injection into at least one lobe of the thymus following thoracotomy, thoracotomy or by injection using a thoracoscope, CT-guided or ultrasound guided percutaneous injection, catheter injection under fluoroscope guidance, or mediastinoscopy.

According to the invention the CD34-positive hematopoietic cells of the donor can be implanted in the transplant recipient prior to transplanting the graft, concurrently with transplanting the graft, or subsequent to the transplantation. Periodic maintenance administration to the thymus may be necessary to maintain adequate levels of tolerance in some patients.

The graft which can be transplanted is a solid organ, tissue or cell collection. The solid organ can be a heart, lung, heart-lung, kidney, pancreas, intestine or liver. A suitable tissue for transplantation according to the present methods is a vessel, heart valve, connective tissue or skin. The transplanted cell collection can comprise CD34-positive hematopoietic cells or cells which express a disease-associated gene product of interest. A microchimerism of donor hematopoietic cells may be detectable in the blood of the transplant recipient for a substantial time after transplant according to the methods described herein.

In another aspect the invention provides a method for producing a gene product of interest in a host mammal in need of said gene product. This aspect comprises implanting in the thymus of the host CD34-positive hematopoietic progenitor cells which encode the gene product of interest. The CD34-positive hematopoietic cells can be genetically engineered to produce the gene product of interest in the recipient host. In one aspect, prior to implanting the modified cells in the host the CD34-positive hematopoietic cells are obtained from the recipient host mammal and

genetically engineered to produce the gene-product of interest. In another aspect the CD34-positive hematopoietic cells can be those obtained from a donor of the same or different species as the recipient and which cells produce the gene product in said donor.

#### Description of the Specific Embodiments

The present invention provides methods and compositions for intrathymic administration of CD34-positive donor cells into a transplant recipient to produce lymphohematopoietic microchimerism that results in prolonged survival of transplanted grafts or organs with or without conventional immunosuppression. Thus, in one aspect the invention provides methods and compositions for inducing tolerance to a donor cell, tissue or organ in a recipient individual. By "tolerance" is meant to refer to an immune response to an allogeneic or xenogeneic graft that is smaller than the immune response that would be observed in an animal which has not received the thymic implant of nonautologous CD34-positive hematopoietic cells, thereby resulting in an extension of graft survival time. The engraftment of donor material into transplant recipients can be accomplished safely and substantially without the risk of graft versus host disease. According to the invention, implantation of donor CD34-positive hematopoietic cells into the thymus provides a level of tolerance in the recipient host to the donor tissue that is sufficient to permit engraftment. Offered by way of explanation but not limitation, it is believed the direct intrathymic injection of CD34-positive hematopoietic donor fractions produces lymphohematopoietic microchimerism and thus a tolerance which prolongs survival of the graft.

The nature of the graft intended for transplantation into a recipient host varies widely. The grafts include, for example, organ transplants, such as heart, heart-lung, lung, kidney, kidney-pancreas, liver, pancreas,

intestines, etc. The grafts can also include tissue transplants, such as of skin, connective tissue, vessels, heart valves, etc. In yet another aspect the grafts can be of cells from a donor, such as bone marrow cells, including the CD34-positive hematopoietic cells which are used to induce tolerance, wherein the CD34-positive hematopoietic cells include stem (progenitor) cells. Other suitable cell collections include pancreatic islet cells, hepatic cells, bone marrow or stem cells. The grafts can also be CD34-positive hematopoietic cells which express a desired protein or chemical or which could be genetically engineered to produce a desired protein or chemical as can be used in treatment of individuals with genetic or acquired deficiencies. The donor CD34-positive hematopoietic cells and grafts can also be xenografts, i.e., from a different species than the recipient, for example, a non-human primate or pig graft can be implanted into a human. As used herein the term "graft" is meant to include the implant in an individual of any non-autologous organ, tissue or cells, unless the context specifically indicates otherwise.

Engraftment of the CD34-positive hematopoietic cells themselves, without a subsequent graft, may also be used to treat genetic or acquired deficiencies. The CD34-positive cells could also be genetically engineered prior to intrathymic implant to produce proteins, chemicals or specific enhancers or suppressors of cell function. Because stem cells may produce progeny, a single application/injection/treatment may be sufficient for the patient's lifetime.

In the present invention the use of CD34-positive hematopoietic cells, substantially depleted of mature T and B lymphocytes, reduces the risk of graft versus host disease in the recipient individual while providing the ability to modify the cellular constituency of the hematopoietic system of the recipient over a prolonged period. The CD34 antigen is present on substantially all hematopoietic precursor cells, but is substantially absent from more mature hematopoietic cells. Within the context of the present invention, CD34-

positive hematopoietic cells include those cells which express the CD34 antigen, among other surface antigens, and include totipotent stem cells as well as committed progenitor cells. The level of expression of the CD34 antigen will vary from one cell type to another. Consequently, a cell is operationally defined as CD34-positive if it expresses sufficient CD34 antigen to be detected by a given method of assay, e.g., by flow microfluorimetry using a fluorescence-activated cell sorter (FACS), by immunofluorescence or immunoperoxidase staining using a fluorescence or light microscope, by radioimmunoassay, or by immunoaffinity chromatography, among numerous other methods which will be readily apparent to one skilled in the art. See, for example, Lansdorp and Thomas, in Bone Marrow Processing and Purging, A.P. Gee (ed.), Boca Raton: CRC Press (1991) pg. 351.

The CD34-positive hematopoietic cells may be obtained from a variety of blood products of the intended donor. As the present invention provides methods for inducing tolerance to the tissue or organ of the donor in the intended recipient, it obviates the necessity of employing as a donor an individual who matches or closely matches the recipient's histocompatibility type. Thus, allogeneic and xenogeneic transplants are made possible by the present invention without employing myeloablation. Alternatively, immunosuppressive regimens can be used which are less toxic to the transplant recipient than those regimens employed in the absence of tolerance to antigens on the donor's CD34-positive cells. As a source of xenografts for humans, baboons have been used in xenograft transplants over the past 20 years with surprising success and, therefore, are considered phylogenetically quite similar to humans, allowing the present invention to be extended to xenograft applications. Other species may also serve as graft donors, such as swine which can serve as a source of, e.g., skin and other tissues for engraftment onto humans.

Sources of CD34-positive cells include bone marrow, peripheral blood, umbilical cord blood, fetal liver, and



spleen of the intended tissue donor. Bone marrow is a particularly rich source of precursor cells (1-2% of marrow), but alternate sources may be preferable because of the discomfort associated with bone marrow aspiration. Bone marrow is typically aspirated from the iliac crest, but may be obtained from other sites (such as the sternum or vertebral bodies) if necessitated by prior or concurrent disease or therapy. In the case of cadaveric donors, vertebral bodies are a convenient source of large quantities of CD34-positive hematopoietic cells.

While peripheral blood contains fewer precursor cells (typically < 1% of peripheral blood mononuclear cells), it is generally easier to obtain than bone marrow. The number of precursor cells circulating in peripheral blood can be increased by prior exposure of the donor to certain growth factors, such as, for example, G-CSF or SCF (KL), and/or certain drugs. For example, antibody to VLA-4, given intravenously, results in release of CD34-positive hematopoietic cells from marrow stores into peripheral blood. Papayannopoulou and Nakamoto, Proc. Natl. Acad. Sci. USA 90:9374-9378 (1993). Thus, administration of an anti-VLA-4 antibody to the donor can facilitate expeditious recovery of CD34-positive cells from peripheral blood. Depending upon the volume which is desired, blood may be obtained by venipuncture or by one or more aphereses on a blood separator.

General methods for separating and processing T- and B-cell-depleted CD34-positive fractions by immunoadsorption to magnetic beads and the like are described in, e.g., Andrews et al., Blood 80: 1693-1701 (1992); Andrews et al., Blood 67:842-845 (1986); Berenson et al., J. Clin. Invest. 81: 951-955 (1988); and Andrews et al., J. Hematol. 2:111-122 (1993), each of which is incorporated herein by reference. Although one can readily separate a bone marrow or peripheral blood specimen or apheresis product into precursor and mature cells, (such as CD34-positive and CD34-negative populations), it is generally preferred to prepare a buffy coat or mononuclear cell fraction from these specimens first,

prior to separation into the respective populations. Methods for the preparation of buffy coats and mononuclear cell fractions are well-known in the art (e.g., Kumar and Lykke, Pathology 16:53 (1984)).

5 Separation of precursor cells from more mature cells can be accomplished by any of a variety of methods known to those skilled in the art, including immunoaffinity chromatography (Basch et al., J. Immunol. Methods 56:269 (1983)), fluorescence-activated cell sorting, panning (Wysocki  
10 and Sato, Proc. Natl. Acad. Sci. USA 15: 2844 (1978)), magnetic-activated cell sorting (Miltenyi et al., Cytometry 11: 231 (1990)), and cytotoxicity. Generally, separation of a heterogeneous population of cells, such as in a bone marrow aspirate or a peripheral blood specimen or apheresis product,  
15 into target (i.e., CD34-positive) and non-target (i.e., CD34-negative) fractions is rarely complete. For the purposes of the present invention, separation and substantial purification or enrichment of CD34-positive hematopoietic cells is considered to have been accomplished if the target  
20 fraction is comprised of at least about 10% CD34-positive cells, typically at least about 50%, more typically at least about 70% CD34-positive hematopoietic cells, preferably at least about 90%, more preferably about 95%, and even more preferably about 98 to 99% or more CD34-positive hematopoietic  
25 cells. In addition, it is desirable to keep the total numbers of mature hematopoietic cells, such as lymphocytes, platelets, granulocytes, and red cells, as low as possible.

The CD34-positive hematopoietic cells may be positively selected or negatively selected. By positive  
30 selection is meant the capture of cells by some means, usually immunological, on the basis of their expression of a specific characteristic or set of characteristics (usually an antigen(s) expressed at the cell surface). For example, CD34-positive cells can be positively selected by any of the  
35 above methods (except cytotoxicity, which would result in destruction of the desired cells) on the basis of their expression of the CD34 antigen utilizing an anti-CD34

antibody, such as the monoclonal antibodies 12.8, My-10, and 8G12 (commercially available from Becton Dickinson Co., Mountain View, CA), or Q-Bend 10 (commercially available from Biosystems Ltd., Waterbeach, Cambridge, England). Although  
5 selection of CD34-positive cells usually involves the use of one or more antibodies or fragments thereof, in some cases selection may involve the use of lectins or other types of receptors or ligands expressed on the cell surface. Among  
10 other antibodies, antigens, receptors and ligands which may be useful, alone or in combination with other markers, for separating CD34-positive cells from CD34-negative cells are transferrin, the transferrin receptor, soybean agglutinin, c-kit ligand, c-kit receptor, HLA-DR, CD33, etc.

Negative selection means the exclusion or depletion  
15 of cells by some means, usually immunological, on the basis of their lack of expression of a specific characteristic or set of characteristics (again, usually a surface antigen). For example, CD34-positive cells can be negatively selected by any of the above methods on the basis of their lack of expression  
20 of lineage-defining antigens, such as CD19 (for B lymphocytes), CD3 (for T lymphocytes), CD56 (for NK cells), etc., utilizing antibodies to the above-mentioned and other lineage-defining antigens. By using a cocktail or mixture of monoclonal antibodies directed to red cell, platelet,  
25 granulocyte, lymphocyte and/or tumor cell antigens, it is possible to leave behind a population of cells which is highly enriched for CD34-positive cells. Numerous monoclonal and polyclonal antibodies suitable for this purpose are known in the art (see, e.g., Leukocyte Typing IV, Knopp et al. (eds.),  
30 Oxford UP, 1989) and are commercially available from a wide variety of sources (for example, Becton Dickinson Co., Mountain View, CA; Coulter Immunology, Hialeah, FL; Ortho Diagnostics, Raritan, NJ, etc.). The CD34-positive hematopoietic cells can also be separated from mature cells by  
35 a combination of negative and positive selection techniques.

The separated CD34-positive hematopoietic cells may be used immediately suspended in an isotonic solution, stored

frozen in a DMSO medium or other suitable freezing medium and thawed at a later date for use, and/or inoculated into a suitable vessel containing a culture medium comprising a conditioned medium and nutritive medium, optionally  
5 supplemented with a source of growth factors and, optionally, human or other animal plasma or serum. If cultured, the resultant cell suspension may be cultured under conditions and for a time sufficient to increase the number of hematopoietic precursor cells relative to the number of such cells present  
10 initially in the blood product. The cells may then be separated by any of a variety of methods, such as centrifugation or filtration, from the medium in which they have been cultured, and may be washed one or more times with fresh medium or buffer. Optionally, the cells may be  
15 re-separated into CD34-positive and -negative fractions, prior to resuspension to a desired concentration in a medium or buffer suitable for injection.

Particularly preferred methods and devices for the selection of CD34-positive hematopoietic cells are described  
20 in U.S. Patent Nos. 5,215,927, 5,225,353, 5,262,334 and 5,240,856, each of which is incorporated herein by reference in its entirety. These patents describe methods and devices for isolating or separating target cells, such as hematopoietic precursor cells, from a mixture of non-target  
25 and target cells, wherein the target cells are labeled, directly or indirectly, with a biotinylated antibody to a target cell surface antigen. Labeled cells are separated from unlabeled cells by, flowing them through a bed of immobilized avidin, the labeled cells binding to the avidin by virtue of  
30 the biotinylated antibody bound to their surface, while the unlabeled cells pass through the bed. After washing the bed material, the labeled (bound) cells can be eluted from the bed, for example, by mechanical agitation. A cell separator device is also provided for separating target cells from  
35 non-target cells, one embodiment being the CEPRATE SC™ cell separation system described in Berenson et al. (Adv. Bone

Marrow Purging & Processings, N.Y.: Wiley-Liss, 1992, pg. 449).

Once isolated, the donor CD34-positive hematopoietic cells can then be introduced into the thymus of the transplant recipient. The thymus provides the CD34-positive cells with an extended half-life, and the implanted cells are treated as "self" by the host, thereby resulting in donor-specific tolerance to the graft. Thoracic organ transplantation provides a convenient opportunity to implant donor marrow components directly into the thymus, which is readily accessible during the cardiac or lung transplant operation. In the absence of thoracic organ transplantation, a small left anterior thoracotomy is performed in the second intercostal space which provides excellent access to the thymus. The purified CD34-positive donor cells, typically suspended in about 1 ml physiologically acceptable medium and any desired markers for locating donor cell injection sites in the thymus, is injected in different portions of at least one lobe and typically both lobes of the thymus. The thoracotomy incision is closed and a chest radiograph can be used to ensure full left lung inflation. Advances in videoscopic, thoracoscopy, mediastinoscopy and CT-guided or ultrasound guides percutaneous procedures make intrathymic delivery of cells possible without thoracic surgery. Since injection of cells is accomplished by simple inoculation through a needle tip, unusual/sophisticated instrumentation is not necessary. Especially in infants, where the thymus is large and the sternum not yet ossified, percutaneous injection can be readily accomplished. In utero injection using ultrasound or fetoscopy is also possible for use of the present methods in fetuses. Further, selective catheterization of thymic veins (via the innominate vein) or arteries allows fluoroscopically-guided injection of cells into the thymus in the radiology suite. Alternatively, CD34-positive hematopoietic cells may be administered via the peripheral blood circulation to the thymus, including the use of agents which target said cells to the thymus.

The tolerizing CD34-positive hematopoietic cells from the donor can be implanted in the thymus of the transplant recipient prior to, at the time of, or shortly following graft transplantation. When given prior to  
5 transplant as in the case of a living donor for bone marrow, kidney, lung, intestine, pancreas, or liver transplant, the CD34-positive cells are typically implanted from one to two weeks prior to graft transplant, more often about two to four weeks prior to transplant, or in some cas s  
10 from four to eight weeks or more prior to transplant. When given prior to the time of transplant the recipient can be assayed for the development of hematopoietic microchimerism such that timing of implantation can be coordinated to achieve levels of tolerance.

15 The level of tolerance can be assessed in the recipient indirectly by determining the level of microchimerism in the blood using markers specific for the donor cells as measured by polymerase chain reaction, etc., as set forth in more detail below. Typically probes can be used  
20 to demonstrate peripheral hematopoietic microchimerism in blood samples from the transplant recipient. The use of PCR analysis and oligonucleotide probes are generally described in, e.g., Erlich et al., Arch. Pathol. Lab. Med. 117: 482-485 (1993); Gaur et al., J. Mol. Evol. 9:599-609 (1992); and  
25 Nuovo, PCR In Situ Hybridization. Protocols and Applications, Raven Press, New York, NY pp 157-183 (1992), each incorporated herein by reference. Probes specific to individual donors are used not only to identify donor cells in recipient peripheral blood, but also to detect donor cells in recipient lymphoid  
30 tissue. A microchimerism typically of at least about 0.01% cells derived from the donor is sufficient to alter immune status in recipients; levels of from about 0.01% to 1.0%, up to 5% or higher are sufficient to establish tolerance to the graft in the recipient. Other methods for measuring tolerance  
35 include acceptance of small patches of donor skin grafts on the recipient skin. A diminished mixed lymphocyte reaction (MLR) of the recipient's lymphocytes to those of the donor may

also be indicative of a level of tolerance. Methods for performing MLRs are well established in the art. However, tolerance to solid organ grafts can occur with intrathymic donor implants even without demonstrable reduction in MLR.

5           As mentioned above, the CD34-positive hematopoietic cells can also be implanted into the thymus at approximately the same time as the graft transplant. This will often occur when the CD34-positive cells are not available in advance, e.g., as with a cadaveric donor. When the graft is a heart,  
10 heart-lung or lung transplant the thymus can be conveniently implanted with the CD34-positive cells during the same surgical procedure.

Donor CD34-positive hematopoietic cells, including stem cells, can also be administered to the graft recipient  
15 post-transplant, either to induce tolerance or to maintain a level of tolerance that was previously established. To induce tolerance the cells are implanted as soon after the transplant as possible, typically within about 1-14 days of the transplant procedure. Percutaneous inoculation of CD34-  
20 positive donor cells into the thymus under CT guidance, or videoscopic introduction of the stem cells, permits intrathymic implantation of cells at a variety of timepoints. Maintenance of a desired level of tolerance may require periodic intrathymic implants of CD34-positive hematopoietic  
25 donor cells in the transplant recipient.

The substantially pure preparation of CD34-positive cells are administered into the thymus in an amount sufficient to induce tolerance in the recipient to the graft being transplanted. This is referred to herein as a "tolerizing"  
30 dose. A tolerizing dose can range from about  $1 \times 10^5$  CD34-positive stem cells up to about  $3 \times 10^8$  cells or more, but more typically from  $5 \times 10^5$  to  $1 \times 10^8$  to induce hematopoietic microchimerism, tolerance and subsequently prolongation of graft survival. Dosing is of logistical importance because it  
35 determines whether a sufficient number of donor CD34-positive hematopoietic cells could be procured from one or two vertebral bodies, ribs, sternum, or iliac crest, all of which

might be available early in an organ donor operation. The equivalent of 2 vertebral bodies of marrow can be processed within 6-10 hours, assuring simultaneous implantation of donor stem cells during the subsequent organ implant operation, if  
5 desired. A requirement for larger doses of stem cells necessitates complete vertebral body marrow procurement following organ harvest and thus may require up to 12-24 hours for processing. In this latter situation, because short  
10 donor heart ischemic times are so critical for cardiac graft function, a cardiac transplant can proceed and the donor marrow fractions are delivered to the recipient after the cardiac transplant. For multi-organ donors, the dose of cells required for engraftment determines whether a single donor can supply enough stem cells for multiple organ transplant  
15 recipients. Up to  $14 \times 10^8$  marrow cells can potentially be recovered from a cadaver donor, with CD34-positive cells more prevalent (at 4.6%) in marrow obtained from vertebral bodies.

The intrathymic administration of CD34-positive hematopoietic cells of the present invention can be employed  
20 without immunosuppressive therapies or in conjunction with such therapies, if desired. Adjunct immunosuppressive therapy may also be employed, i.e., chemical (e.g., cyclosporin A, steroids, and/or azathioprine) anti-lymphocyte globulin or serum, or radiological myeloablation, and may be at levels  
25 less than those which would be typically employed in the absence of intrathymic implantation of CD34-positive hematopoietic stem cells. Other therapies can also accompany the present methods to facilitate tolerance and/or acceptance of the graft, such as, e.g., OKT3, FKS06, other  
30 immunosuppressive drugs, and therapies to reduce humorally mediated rejection such as photophoresis, plasmapheresis, soluble complement fractions, cobra venom factor, genetic manipulation of donor organ endothelium or the complement activation cascade, methods for downregulating VCAM-1 or E-selectin expression or cytokine production and the like.  
35

In another aspect of the invention donor CD34-positive hematopoietic cells intended for intrathymic implant



can be genetically engineered to encode a gene product of interest. Methods for retroviral transduction of stem cells are described in, e.g., Emery et al., Blood 81:2460-2465 (1993), which is incorporated herein by reference.

5           The following examples are offered by way of illustration, not by way of limitation.

#### EXAMPLE I

10

This Example describes the intrathymic inoculation of CD34-positive marrow fractions for the induction of hematopoietic microchimerism and prolongation of skin allograft survival.

15

Initial studies were designed to assess the production of hematopoietic microchimerism by direct introduction into the thymus of a CD34-positive hematopoietic cell-rich donor marrow fraction defined by an anti-CD34 antibody, 12.8. CD34-positive hematopoietic cells were isolated from marrow specimens obtained from adult male donor baboons and enriched to approximately greater than 98% pure according to the procedure described in Andrews et al., Blood 80:1693-1701 (1992), incorporated herein by reference. Four weaned juvenile female baboons, ranging from 6-12 weeks of age (1-2 kilograms), served as recipients. Donor and recipient baboons were selected at random from the primate breeding colony (Medical Lake, WA) and were unrelated. Through a left anterior thoracotomy under general anesthesia,  $1 \times 10^6$  donor stem cells were injected into 4-5 sites in the left lobe of each recipient thymus and each injection site was marked with a surface clip.

20

Peripheral blood obtained from each recipient was examined for the presence of male donor cells, detected by PCR amplification of a Y chromosome-specific (male-specific) determinant as described in Reitsma and Harrison, Cytogenet. Cell Genet. 64:213-216 (1993), incorporated herein by reference. This assessment was made more than twice on each

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blood and marrow specimen, several months apart, to confirm the reproducibility of PCR results.

Two animals were treated with standard triple drug immunosuppression, consisting of cyclosporin A (sufficient to  
5 maintain whole blood HPLC levels at 300-500 ng/dl),  
dexamethasone (0.5/mg/kg/day), and azathioprine, 3 mg/kg/day.  
The two control animals, also with CD34-positive hematopoietic  
cell injections, received no immunosuppression.

The two immunosuppressed recipients developed  
10 peripheral blood microchimerism within two weeks of donor cell  
injection. In both animals, microchimerism as evidenced by  
PCR amplification of the male-specific determinant persisted  
for at least 16 months from the time of donor cell  
implantation (Table 1). In the two recipients without  
15 immunosuppression, microchimerism developed even earlier, at  
three days following donor cell implantation in one animal, at  
one week in the other. Microchimerism persisted for at least  
6 months in one animal, and 9 months in the other.

Table 1

	<u>Sample</u>	<u>WKS Post Injection</u>	<u>M92522</u>	<u>F93011</u>	<u>F92520</u>	<u>M92523</u>
5	PBL	1	3/3	2/3	0/3	1/3
	PBL	2	2/3	ND	1/3	1/3
	PBL	3	3/4	3/4	ND	ND
	PBL	4	ND	3/3	1/3	2/4
10	PBL	5	3/4	3/4	2/4	3/4
	PBL	6	0/3	2/3	0/3	2/4
	PBL	7	3/4	3/3	0/3	0/3
	PBL	8	1/2	1/2	0/3	2/3
	PBL	9	4/4	0/2	0/3	1/3
15	PBL	10	1/2	ND	0/3	1/3
	PBL	11	1/3	ND	ND	ND
	PBL	17-19	1/3	ND	1/3	3/3
	BM	17-19	0/2	ND	0/3	2/3
	PBL	20-22	0/2	1/3	1/2	1/2
20	CD2+PBL	20-22	0/2	1/2	2/3 [6*10 <sup>4</sup> ]	2/2
	CD20+PBL	20-22	0/2	0/1	2/2 [5*10 <sup>3</sup> ]	1/2
	PBL	23-25	1/2	2/3	2/3	ND
	PBL	26-28	0/2	0/1	0/6	1/2 [7*10 <sup>4</sup> ]
	CD2+PBL	26-28	0/2	1/1 [4*10 <sup>4</sup> ]	4/5 [1*10 <sup>4</sup> ]	1/2 [7*10 <sup>3</sup> ]
25	CD20+PBL	26-28	0/2	0/1 [5*10 <sup>3</sup> ]	0/5	1/2 [10 <sup>3</sup> ]
	PBL	27-29	0/2	0/1	0/6	1/2
	CD34+BM	27-29	0/1	0/2	0/2	1/2 [10 <sup>4</sup> ]
	PBL	46-48	0/1	ND	0/1	0/1
	BM	46-48	1/1	ND	ND	ND
30	PBL	60-62	0/1	0/1	1/1	1/1

Replicate samples of unseparated PBL and BM (10<sup>3</sup> /sample) and double sorted CD2+ (T cells), CD20+ (B cells) from blood and CD34+ cells from marrow were assayed by PCR. Based on limiting dilution analysis of normal male PBL in normal female PBL the sensitivity of the PCR analysis is 1 male cell in 1,000 to 10,000 female cells. Numbers in [] represent the number of cells in each sample tested if different from 10<sup>3</sup>. Animals F92520 and M92523 were immunosuppressed with dexamethasone, azathioprine, and cyclosporin A at the time of intrathymic injection of cells and remained on immunosuppressive drugs after injection.

ND= not done

In the studies on baboons with intrathymic implantation of donor cells, none of the six recipients given intrathymic purified CD34-positive cells, without myeloablation, developed any overt evidence of graft versus host disease, with follow-up out to 16 months. In these animals with intrathymic implants, skin, liver, and mucosal tissues have not yet been examined histologically to look for graft versus host disease, so lack of graft versus host

disease can only be inferred from the apparent lack of symptoms.

These results demonstrate that stable hematopoietic microchimerism can be achieved by the introduction of donor cells into the thymus. This method uses purified CD34-positive cell fractions which are directly introduced into the thymus to achieve engraftment. The microchimerism was accomplished using standard triple drug immunosuppression without host T cell depletion, and was also accomplished without immunosuppression and without host T cell depletion. Further, microchimerism was achieved with a smaller dose of donor cells than has commonly been reported for other settings. Importantly, graft versus host disease has not yet been observed. As the studies were performed in a primate model, the results are directly relevant to the clinical applicability of the methods to humans.

#### EXAMPLE II

##### Intrathymic Stem Cell Implantation Prolongs Skingraft Survival

This Example demonstrates that intrathymic CD34-positive hematopoietic cell implantation and/or development of microchimerism prolongs survival of grafts from the cell donor.

Skin grafts were used as a test of donor-specific tolerance because skin grafts express non-MHC antigens which are not tolerized by hematopoietic cells and thus are a more difficult model of allograft acceptance. Nakafusa et al., Transplantation 55:877-882 (1993); Reitsma and Harrison, supra, Steinmuller and Lofgreen, Nature 248:796-798 (1974); and Ildstad et al., J. Surg. Res. 51: 372-376 (1991). Demonstration of prolongation of skin graft survival in this model is predictive of acceptance of solid organ grafts.

As in Example I, two juvenile female baboons were injected intrathymically with  $1 \times 10^6$  CD34-positive marrow cells from an unrelated male donor baboon. Unlike Example I, CD34-

positive cells were injected into both lobes of the recipient thymus. Because chimerism had developed earlier in the unimmunosuppressed animals in the previous study, neither of these two recipients was given immunosuppression. Using PCR amplification of the male-specific sequence, donor cells were detectable in the blood and marrow of both animals after transplantation, demonstrating hematopoietic chimerism.

Two months following injection of CD34-positive cells, both recipient animals had full thickness 6mm. diameter skin grafts placed on their posterior thorax. Nine grafts were placed, three of autologous skin, three from the male donor of the CD34-positive cells, and three from another unrelated baboon. (Each recipient animal served as the unrelated control for the other.)

All third-party allogeneic grafts were rejected (sloughed) in 24 days. In contrast, skin grafts from the marrow cell donor persisted for 42 days in both animals. Autologous grafts were accepted indefinitely and incorporated. A biopsy from the CD34-positive cell-donor graft in one recipient confirmed the presence of donor cells in this skin (by PCR amplification of DNA isolated from the tissue) on post-transplant day 27, three days after the sloughing of the third-party graft, providing further proof of specific prolongation of skin graft survival from the stem cell donor but not the third-party donor.

These results confirm that even in the absence of immunosuppression implantation of CD34-positive donor hematopoietic cells in the thymus produces hematopoietic microchimerism and is associated with prolongation of skin allograft survival that is specific for the marrow donor.

The tissue surrounding the CD34-positive cell-donor skin graft became somewhat indurated, a phenomenon not seen in either the autologous or the third-party allografts. This finding may be related to incomplete tolerance to non-MHC antigens expressed in the skin, or to "auto-immune" type phenomena resulting from humoral antibodies developing either to the stem cell donor or, perhaps, to new cross-reacting

alloantigens introduced by the third-party allograft. Donor-specific alloantibodies did develop after skin grafting (see below) but did not affect chimerism.

Excisional biopsy performed on one row of grafts from each animal at 27 days post grafting revealed hypertrophic epidermis and intradermal fibrosis in the cell-donor graft, resembling the changes of scleroderma. Compared to the third-party allograft on the same day, less lymphocytic infiltration was seen in the graft derived from the cell donor.

In these healthy, unimmunosuppressed recipients, ingrowth of recipient tissue into the margins of these skin allografts was quite rapid. To rule out simple displacement of the skin graft by proliferating recipient cells at the graft margins, rather than true rejection, the CD34-positive cell-donor and third-party skin allografts were repeated, using larger, 1.3 cm. diameter grafts. Consistent with the previous observations, the grafts from the CD34-positive cell donor evidenced specific prolongation of survival as compared to third-party grafts, which were rejected at 25 days.

The repeat third-party skin allografts were not rejected in an accelerated fashion. This finding, as well as the survival of the first set of third-party allografts to 24 days, is indicative of a non-specific suppression of alloreactivity associated with microchimerism and/or intrathymic CD34-positive hematopoietic cell engraftment. Alternatively, the two recipient baboons, who each served as third-party skin donors for the other, may have inadvertently shared some key MHC antigens with the recipient. The latter possibility is unlikely since the recipient demonstrated full in vitro alloreactivity to this third-party donor on mixed lymphocyte culture.

The presence of alloantibody to donor antigens introduced in the thymus was determined using a two-color immunofluorescence technique and analyzed on a flow cytometer. Antibody in recipient serum binding to donor cells but not autologous cells or cells from the other recipient was

interpreted as antibody to donor alloantigens. As shown in Table 2, in recipients of allogeneic CD34-positive hematopoietic cells and skin grafts, both recipients developed antibody to the cell donor. Results from a serum sample drawn after skin grafting are also shown. The animals identified as RS and RU are the CD34-positive cell recipients. Animal 90221 is the cell donor. These studies demonstrate that alloantibody production was stimulated by the donor skin graft in these two unimmunosuppressed recipients, even though both demonstrated peripheral blood microchimerism at this point in time. Donor-specific alloantibodies did not prevent prolongation of hematopoietic microchimerism or skin graft survival from this same donor baboon.

Table 2: IgG Antibody Binding to T Lymphocytes

<u>Serum Tested</u>	<u>90221</u>	<u>RS</u>	<u>RU</u>
90221	Negative	Negative	Negative
HLA class I MAb	Positive	Positive	Positive
RS- Pre-stem cell transplant	Negative	Negative	Negative
RS- 10 days post skin transplant	Positive	Negative	Negative
RU- Pre-stem cell transplant	Negative	Negative	Negative
RU- 10 days post skin transplant	Positive	Negative	Negative

### EXAMPLE III

#### Intrathymic Implant For Xenografts

This Example describes the use of the present invention in primate xenograft transplantation by demonstrating that intrathymic injection of CD34+ fractions of human donor marrow can induce hematopoietic microchimerism in baboon recipients without immunosuppression. A human donor-to-baboon recipient protocol was used as a model for baboon-

to-human concordant xenograft organ (liver, kidney, heart, etc.) transplants.

The methodology for both donor and recipient xenograft procedures was generally that set forth above in Examples I and II for the baboon allografts. Human CD34-positive donor cells were isolated from donor marrow obtained from human male cadaver tissue donors with appropriate familial consent. Human cadaver marrow specimens were cryopreserved and separated into CD34+ fractions (98% pure) by the same methods used in the baboon allograft Examples. Human donor lymphocytes were HLA typed and frozen for future in vitro studies. Split thickness skin grafts were harvested from the same human cadaver donor and cryopreserved, the only variation from the baboon allografts in which full thickness skin grafts were utilized.

The two recipient baboons for the xenografts were both juvenile females (10 to 12 weeks of age), as in the allograft Examples. Recipient serum was drawn pre-transplant to assess the presence of preformed anti-human antibodies.  $6.5 \times 10^5$  human CD34-positive donor hematopoietic cells were injected into 4 sites in both lobes of the recipient thymus, using a left thoracotomy approach. One recipient was treated with standard triple drug immunosuppression (cyclosporin A, azathioprine, and steroids); the other recipient animal was not immunosuppressed. Peripheral blood samples were taken at intervals to assess the development of hematopoietic microchimerism, as assayed by PCR amplification of human Y chromosome determinants. Serum was taken to assess the development of xenoantibodies.

At three months after CD34-positive human hematopoietic cell transplantation, skin grafts were placed on both recipients from both the human xenograft marrow donor and from an unrelated baboon allograft skin donor. Grafts were biopsied at 10, 18, and 28 days for PCR analysis to assess the presence of donor DNA.

Both the recipient baboons developed peripheral blood microchimerism with human donor cells, demonstrable by



PCR techniques up to 8 months following stem cell implantation, which still persist. Human male donor cells were identified at frequencies of at least 1:500 in both CD34- and CD34+ fractions and in both CD3+ and CD3- fractions, suggesting that the original implanted stem cells had produced progeny in both T-lymphocytic as well as other cell lines.

As shown in Table 3, xenoantibodies were not present in these juvenile baboons prior to CD34-positive hematopoietic cell transplantation. The animal receiving human CD34-positive hematopoietic cells without immunosuppression (SD) developed antibody to cells from the human following hematopoietic cell implantation. The animal that received human CD34-positive hematopoietic cells with triple therapy immunosuppression (SC) did not develop antibody to donor cells after stem cell infusion but did make antibody following challenge with a skin graft from the stem cell donor.

Table 3

Xenoantibody data: <u>Serum Tested</u>	IgG antibody binding to T <u>lymphocytes from: Human donor</u>
Human serum-negative control	Negative
Human alloimmune serum-positive control	Positive
Baboon SD-pre stem cell transplant	Negative
Baboon SE-post stem cell transplant	Positive
Baboon SD-post skin graft	Positive
Baboon SC-pre stem cell transplant	Negative
Baboon SC-post stem cell transplant	Negative
Baboon SC-post skin graft	Positive

The development of anti-human antibodies did not affect the persistence of hematopoietic microchimerism.

5 The host response to the human cell donor xenograft skin was quite different than to the skin allograft from the unrelated baboon. The human xenograft (stem cell donor) appeared pink and vascularized as compared to the necrotic baboon allograft in the immunosuppressed animal (SC) at 22 days posts skin grafting.

10 The finding of hematopoietic chimerism in human-to-baboon concordant xenografts establishes that intrathymic implantation of donor CD34-positive hematopoietic cells are useful in the induction of tolerance across xenograft as well as allograft barriers.

15 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1           1. A composition for enhancing engraftment of a graft  
2     in a transplant recipient wherein the graft is obtained from a  
3     donor who is different from the transplant recipient, which  
4     comprises a collection of CD34-positive hematopoietic cells  
5     isolated from the donor, formulated in amount sufficient to  
6     establish tolerance to the graft and suitable for implantation  
7     in the thymus of the transplant recipient.

1           2. The composition of claim 1, wherein the CD34-  
2     positive hematopoietic cells are obtained from the bone marrow  
3     of the graft donor.

1           3. The composition of claim 1, wherein the CD34-  
2     positive hematopoietic cells are obtained from the blood of  
3     the graft donor.

1           4. The composition of claim 1, wherein the CD34-  
2     positive hematopoietic cells are at least about 98% pure.

1           5. The composition of claim 1, wherein the cell  
2     collection comprises CD34-positive hematopoietic cells,  
3     pancreatic islet cells, hepatic cells, bone marrow or stem  
4     cells, or cells which express a disease-associated gene  
5     product of interest.

1           6. The composition of claim 1, wherein approximately 5  
2     x 10<sup>5</sup> to 1 x 10<sup>8</sup> CD34-positive hematopoietic cells are  
3     formulated for implantation in the thymus of the transplant  
4     recipient.

          7. The composition of claim 1, which is formulated in a  
form suitable for injection into the thymus by thoracoscope,  
CT-guided or ultrasound guided percutaneous injection,  
catheter injection under fluoroscope guidance, or  
5     m diastinoscopy.

1           8. A method for enhancing engraftment in a transplant  
2 recipient of a graft obtained from a donor who is different  
3 from the transplant recipient, comprising:

4           implanting in the thymus of the transplant recipient  
5 CD34-positive hematopoietic cells obtained from the donor in  
6 amount sufficient to establish tolerance to the graft and  
7 thereby enhance engraftment of the graft in the transplant  
8 recipient.

1           9. The method of claim 8, wherein the CD34-positive  
2 hematopoietic cells are obtained from the bone marrow of th  
3 graft donor.

1           10. The method of claim 8, wherein the CD34-positive  
2 hematopoietic cells are obtained from the blood of the graft  
3 donor.

1           11. The method of claim 8, wherein the CD34-positive  
2 hematopoietic cells are contained in a preparation that is at  
3 least about 98% pure for CD34-positive hematopoietic cells.

1           12. The method of claim 8, wherein the CD34-positive  
2 hematopoietic cells are implanted in the transplant recipient  
3 prior to transplanting the graft.

1           13. The method of claim 8, wherein the CD34-positive  
2 hematopoietic cells are implanted in the transplant recipient  
3 concurrently with transplanting the graft.

1           14. The method of claim 8, wherein the CD34-positive  
2 hematopoietic cells are implanted in the transplant recipient  
3 subsequent to transplanting the graft.

1           15. The method of claim 8, wherein the graft is a solid  
2 organ, tissue or cell collection.

1           16. The method of claim 15, wherein the organ is a  
2 heart, lung, heart-lung, kidney, pancreas, intestine or liver.

1           17. The method of claim 15, wherein the tissue is a  
2 vessel, heart valve, connective tissue or skin.

1           18. The method of claim 14, wherein the cell collection  
2 comprises CD34-positive hematopoietic cells, pancreatic islet  
3 cells, hepatic cells, bone marrow or stem cells, or cells  
4 which express a disease-associated gene product of interest.

1           19. The method of claim 8, wherein the graft is an  
2 allograft.

1           20. The method of claim 8, wherein the graft is a  
2 xenograft.

1           21. The method of claim 20, wherein the graft is  
1 obtained from a non-human primate or swine and the transplant  
2 recipient is a human.

1           22. The method of claim 8, wherein approximately 5 x  
2 10<sup>5</sup> to 1 x 10<sup>8</sup> CD34-positive hematopoietic cells are implanted  
3 in the thymus of the transplant recipient.

1           23. The method of claim 8, wherein said implanting is  
2 by injection into at least one thymus lobe following  
3 thoracotomy.

1           24. The method of claim 8, wherein said implanting is  
2 by injection into at least one thymus lobe by thoracoscope,  
3 CT-guided or ultrasound guided percutaneous injection,  
4 catheter injection under fluoroscope guidance, or  
5 mediastinoscopy.

1           25. The method of claim 8, wherein said tolerance in  
2 the transplant recipient to the graft is sufficient to reduce  
3 or eliminate the need for chemotherapeutic immunosuppression  
4 or myeloablative treatment of the transplant recipient.

1           26. The method of claim 8, comprising further treating  
2 said graft recipient with dosages of immunosuppressive  
3 chemotherapy which are less than those required for transplant  
4 recipients who have not received implantations of donor-  
5 derived CD34-positive hematopoietic cells.

1           27. The method of claim 8, wherein a microchimerism of  
2 donor hematopoietic cells is detectable in the blood of the  
3 transplant recipient.

1           28. A method for producing a gene product of interest  
2 in a host mammal in need of said gene product, comprising:  
3           implanting in the thymus of said host CD34-positive  
4 hematopoietic progenitor cells which encode the gene product  
5 of interest.

1           29. The method of claim 28, wherein the CD34-positive  
2 hematopoietic cells are genetically engineered to produce the  
3 gene product of interest in the recipient host.

1           30. The method of claim 29, further comprising, prior  
2 to said implanting step, the steps of:  
3           obtaining CD34-positive hematopoietic cells from the  
4 recipient host mammal, and  
5           genetically engineering the CD34-positive hematopoietic  
6 cells from the recipient host mammal to produce the gene-  
7 product of interest.

1           31. The method of claim 28, wherein the CD34-positive  
2 hematopoietic cells are obtained from a donor of the same  
3 species as the recipient and said cells produce said gene  
4 product in said donor.

1           32. The method of claim 28, wherein the CD34-positive  
2 hematopoietic cells are obtained from a donor of a different  
3 species as the recipient and said cells produce said gene  
4 product in said donor.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/14773

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 35/12, 35/28, 48/00; C12N 5/08, 5/10

US CL :514/44; 435/240.1, 240.2; 424/93.1, 93.21

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/240.1, 240.2; 424/93.1, 93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS, MEDLINE, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	WO, A, 95/03062 (CELLPRO) 02 February 1995, see entire document.	1-32
Y	Blood, Volume 84, Number 8, issued 15 October 1994, CHEN et al., "Engraftment of Human Hematopoietic Cells With Secondary Transfer Potential in SCID-hu Mice", pages 2497-2505, see entire document.	1-32
Y	Blood, Volume 81, Number 9, issued 01 May 1993, EMERY et al., "Retrovirus-Mediated Transfer and Expression of an Allogeneic Major Histocompatibility Complex Class II DRB cDNA in Swine Bone Marrow Cultures", pages 2460-2465, see entire document.	1-32

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

•	Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A	document defining the general state of the art which is not considered to be of particular relevance		
E	earlier document published on or after the international filing date	X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O	document referring to an oral disclosure, use, exhibition or other means		
P	document published prior to the international filing date but later than the priority date claimed	A	document member of the same patent family

Date of the actual completion of the international search

21 JANUARY 1996

Date of mailing of the international search report

28 FEB 1996

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/14773

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Blood, Volume 84, Number 5, issued 01 September 1994, PLUM et al., "Human CD34+ Fetal Liver Stem Cells Differentiate to T Cells in a Mouse Thymic Microenvironment", pages 1587-1593, see entire document.	1-32
Y	Blood, Volume 84, Number 5, issued 01 September 1994, AKKINA et al., "Modeling Human Lymphoid Precursor Cell Gene Therapy in the SCID-hu Mouse", pages 1393-1398, see entire document.	1-32
X, P	Blood, Volume 84, Number 10, Supplement 1, issued 15 November 1994, ALMIEDA-PORADA et al., "Co-Transplantation Of Autologous Stromal Cells With Purified Adult Human Hematopoietic Stem Cells (HSC) Results in Increased Engraftment And Early Donor Cell Expression In Sheep", Abstract 996, see entire document.	1-32
X, P	Blood, Volume 84, Number 10, Supplement 1, issued 15 November 1995, Andrews et al., "Intrathymic Injection Of Purified Allogeneic CD34+ Marrow Cells Produces Hematopoietic Microchimerism In Baboons In The Absence Of Immunosuppression And Myeloablation", Abstract Number 998, see entire document.	1-32
Y	Journal of Experimental Medicine, Volume 174, issued November 1991, PEAULT et al., "Lymphoid Reconstitution of the Human Fetal Thymus in SCID Mice with CD34+ Precursor Cells", pages 1283-1286, see entire document.	1-32
Y	Journal of Experimental Medicine, Volume 175, issued April 1992, VANDEKERCKHOVE et al., "Human Hematopoietic Cells and Thymic Epithelial Cells Induce Tolerance via Different Mechanisms in the SCID-hu Mouse Thymus", pages 1033-1043, see entire document.	1-32